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THE RESISTANCE OF TUBIFICID WORMS TO THREE COMMON POLLUTANTS

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(with 3 figs.)

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The Resistance of Tubificid Worms to Three Common Pollutants*

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(with 3 figs.)

Introduction and Literature Review

Aquatic worms of the family Tubificidae have interested aquatic biologists for many years. The family Tubificidae was characterized by Stephenson (1930) as 'small aquatic worms, up to 200 mm in length, with setae in four bundles per segment, with usually an interminate number of setae per bundle.' The family Tubificidae according to present systematic studies contains approximately eighteen genera. Representatives of two of the genera, *Limnodrilus* and *Tubifex* are the most commonly found types. They differ in their setae and the structure of their reproductive systems and thus these are the major taxonomic characters used in their separation.

Tubificids are often associated with pollutional environments. They are quite resistant to most pollution factors and in areas wherein all other organisms have been killed off or have vacated the area, the tubificids proliferate and thrive. Apparently the removal of their natural enemies allows their populations to explode and they reach population densities of several thousand individuals per square meter of bottom area. It is most interesting that in such conditions the great majority of the population consists of *Tubifex tubifex* (Müller) and *Limnodrilus hoffmeisteri* Claparede, present in about equal numbers. The closeness of their relationship has

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fostered several questions as to the nature of their physiological and ecological interactions. Studies are currently underway in an attempt to better understand these relationships.

Tubificids have a reputation for resistance to pollutants. The common name of 'sludge worms' attests to that. However, no quantitative work on the extent of this resistance has been carried out. For a term such as 'tolerant' to have meaning, some quantification must be carried out under standard laboratory procedures. This report presents some data from such procedures and is an attempt to quantify the tolerance of tubificid worms to three common types of pollutants, the metals, lead and zinc and the wood preservative, sodium pentochlorophenol. These materials were selected because of their common occurrence in streams as pollutants and because they have been used in similar investigations using fish as the test animal, ERICHSEN JONES (1936), CARPENTER (1927), WESTFALL (1945) and CRANDALL (1960). A number of similar studies were reviewed by DOUDOROFF and KATZ (1950—1953).

MATERIALS AND METHODS

Worms were collected from local streams in bottom material which was washed away in the laboratory with a stream of water and a set of standard sieves. The sieves were placed in water in white pans and the worms hand sorted. The worms were then placed in small finger bowls containing 100 ml of modified Knop solution[1], and buffered to a pH of 7.50. They were kept in this manner until used, a period of not more than three days. The dishes were maintained at a constant temperature of 20° C. The test solutions were made from a modified Knop solution base to which was added lead nitrate, ($PbNO_3$), zinc sulfate, ($ZnSO_4$) or sodium pentochlorophenate ($Na_2C_6Cl_5OH$), whichever solution was wanted. The substances were measured in such a way that a 1 part per million (ppm) solution actually contained 1 mg of the metal per liter of modified Knop solution. The solutions thus prepared were added to the finger bowls in 100 ml quantities. The pH level desired was obtained by buffering with CLARK's phosphate buffer solution prepared as needed.

Each experiment consisted of ten dishes containing 100 ml of the test solution and ten worms. In the first experiments *Tubifex* and

[1]) Knop Solution is prepared from $Ca(NO_3)_2$, KNO_3 and $MgSO_4$ as described by BEERS in the recent edition of Culture Methods for Invertebrate Animals by NEEDHAM, GOLTSOFF, LUTZ & WELCH (1959).

Limnodrilus were carefully separated and treated in parallel fashion. This proved unnecessary, however, since both gave the same reactions to various solutions and so the separation was dropped. Instead frequent determination of the approximate percentage of each in the test populations was run to assure the improbability of getting other worms into the experiments. The dishes containing the test solution and worms were placed in a constant temperature chamber at a temperature of 20° C for a period of twenty four hours. At the end of that time the dishes were removed and the number of worms surviving was determined. The general appearance of the worms was noted. These data were treated statistically to determine the effect of each pollutant. Median tolerance limits (TL_m) were determined from the data.

Table 1

Effects of pH on the survival of tubificids

pH	% Survival
3.0	0.0
4.2	0.0
5.5	24.0
6.0	77.0
7.0	93.0
7.5	96.0
8.0	94.0
9.0	81.0
10.0	38.0
11.0	0.0

Results

In preliminary studies the effect of pH on the worms was determined. The results of the effect of pH on the tubificids in modified Knop solution are demonstrated in table 1. It will be noted that between the pH levels of 7.0 and 9.0 the worms lived quite well. These results are representative of several thousands worms for periods of up to 72 hours duration. Application of these data in the manner described by Henderson & Tarzwell (1957) allows determination of the Tl_m. From fig. 1 it may be noted that the pH levels of 5.8 and 9.7 are the calculated levels at which 50% of the worms survive. This should not be taken to indicate the pH tolerance of tubificids in general, but only for their tolerance in Knop solution and at 20° centigrade.

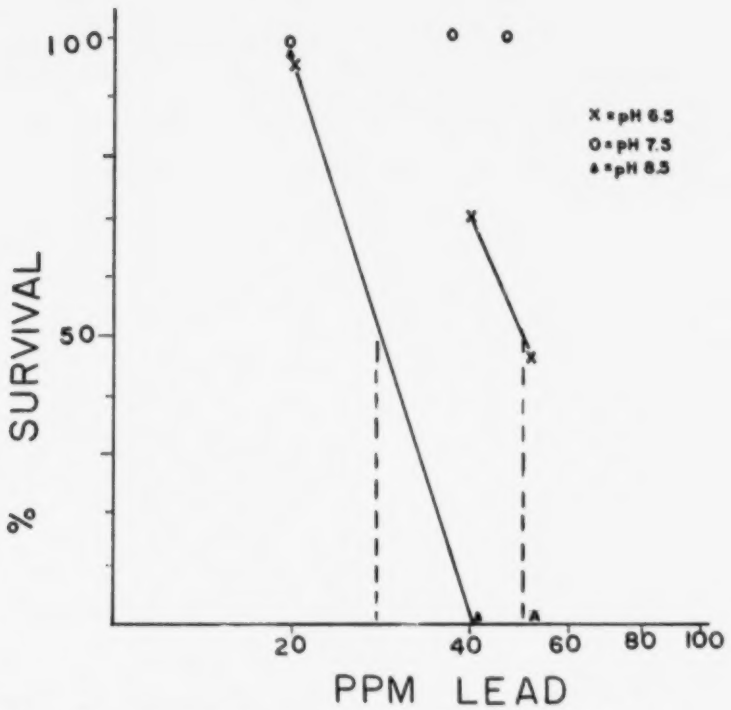

Fig. 1. The survival of tubificids exposed to concentrations of lead.

The results from the experiments in the determination of lead toxicity are listed in Table 2. These data are interpreted as the calculated Tl_m in fig. 2. In this figure the Tl_m of 27.5 ppm lead at a pH

Table 2

The effects of various concentrations of lead on the survival of tubificids

	ppm lead			
	10	20	40	50
pH 6.5	58%	92%	68%	48%
pH 7.5	100%	97%	100%	98%
pH 8.5	94%	93%	0%	0%

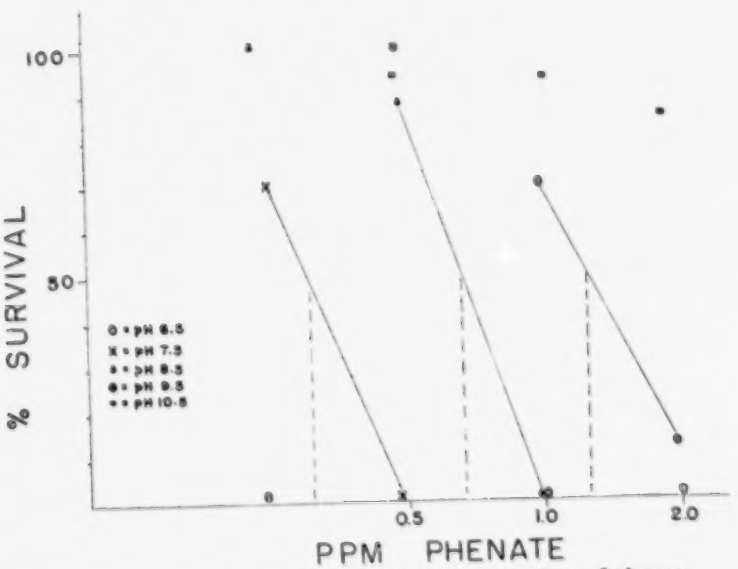

Fig. 2. The survival of tubificids exposed to concentrations of phenate.

of 8.5 is the lower limit and 49.0 ppm at a pH of 6.5 is the upper limit. At concentrations of greater than 50 ppm the lead precipitated from the solution and higher levels were not run for that reason.

The results of the experiments concerning the effect of phenate are shown in Table 3. It will be noted that the Tl_m of phenate is much lower than the zinc or lead. Its apparent interaction with the pH is quite noticeable. The influence of a neutral environment, (ph 7.5)

Table 3

The effects of various concentrations of sodium pentachlorophenate on the survival of tubificids

	ppm sodium pentachlorophenate			
	0.25	0.50	1.0	2.0
6.5	0%	0%	0%	0%
7.5	71%	0%	0%	0%
pH 8.5	100%	89%	0%	0%
9.5	100%	100%	70%	12%
10.5	100%	96%	94%	85%

lowers the Tl_m to 0.31 ppm whereas in a slightly alkaline environment, (pH 8.5) the Tl_m is doubled. 0.67 ppm. However in an alkaline environment (pH 9.5) the Tl_m of 1.4 ppm is again about double the previous figure. This indicates a considerable effect by the pH on the toxicity of phenate.

The effects of the concentrations of zinc are shown in Table 4. These results show the most limited reaction levels of all of the toxicants used. This is reflected in the calculation of the Tl_m in fig. 3.

Due to the fact that at pH levels above and below a neutral pH of 7.5 less than 50% of the populations survived the Tl_m for zinc is calculated only for the pH of 7.5. It is 46 ppm at that pH.

All data were treated statistically after the methods of SIMPSON, ROE & LEWONTIN (1960) and WINER (1962) and an analysis of variance (ANOV) was determined. The results of the ANOV are shown in table 5. The data show a reliability at the 0.005 level

Table 4

The effects of various concentrations of zinc on the survival of tubificids

	ppm zinc					
	10	20	30	40	50	70
6.5	32%	13%	18%	9%	8%	4%
pH 7.5	100%	94%	88%	83%	31%	38%
8.5	43%	20%	0%	0%	0%	0%

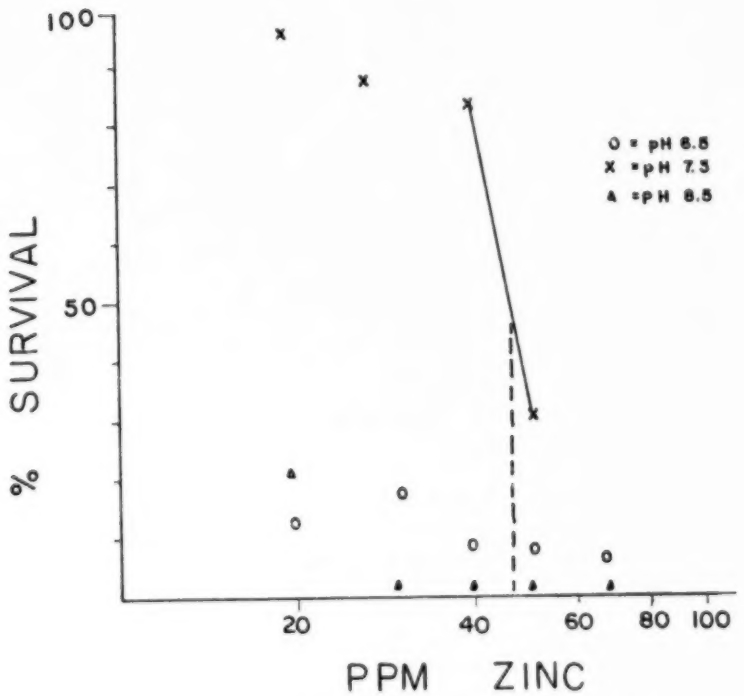

Fig. 3. The survival of tubificids exposed to concentrations of zinc.

Table 5

Summary table for the analysis of variance for pollutants

Source	S.S.	DF	Mean square	F
Lead Experiments				
pH	16203.8	2	8101.9	16.48**
lead	10731.5	3	3577.2	7.28**
interaction	19572.4	6	3262.1	6.64**
Phenate Experiments				
pH	68733.8	4	17183.5	198.5**
phenate	27722.6	3	9240.9	106.8**
interaction	32167.2	12	2680.6	30.9**
Zinc Experiments				
pH	44148.2	2	22074.1	91.0**
zinc	13483.5	5	2696.7	11.1**
interaction	5400.5	10	540.5	2.23*

* Significance at 0.05 ** Significance at 0.005

except for the interaction of zinc and pH which has a reliability level of 0.05. These figures reinforce the value of the data presented.

Discussion

Lead

The data of Table 5 show that the influence of lead alone, and with the synergestic effect of pH has a toxic effect on tubificids. The mechanism of action of the toxic quality of lead is not specifically known for tubificids. The nitrate present in the solutions as lead nitrate can be discounted according to the work by Erichsen Jones (1941). Worms killed by the lead solutions began to degenerate rather quickly. A white precipitate covered the body and upon close examination this was found to apparently be a precipitated mucous covering. The data of Carpenter (1927) and Erichsen Jones (1936)

would support the formation of such a mucous precipitate. WESTFALL (1945) showed the disruption of oxygen exchange across the gills of fish due to the formation of a lead-mucous precipitate. In tubificids the epidermis has many mucous cells which could supply the necessary secretion for the precipitation reaction. In these worms it is known that the process of oxygen exchange occurs through the body wall. Thus the data available would tend to point to the interference of the oxygen exchange by the precipitated mucous as the mechanism responsible for the lethal effect of lead in tubificids. The effects of pH, above or below normal may affect the ionization of the lead or the mucous molecules in such a manner as to alter the volume of precipitation and in this way alter the toxic qualities of a concentration of lead, whatever it may be.

The effect of lead as an internal poison can be greatly discounted since the damage to fish reported by CRANDALL (1960) would require greater time than the 24 hour limit of this study.

The toxic levels for lead of 27.5—49.0 ppm with variance in pH are considerably greater than the amounts of lead which were fatal to a number of fish in earlier studies as reviewed by DOUDOROFF & KATZ (1953). As pointed out by DOUDOROFF & KATZ, the amount of calcium present, the pH of the water, as well as total hardness will affect the level of lead necessary to kill fish. Of these factors only the pH was varied in this study because of the fixed content of the solution. The form of the lead also may be a variable with lead nitrate, as used in this study, being more toxic than lead acetate or lead sulfate. In any case, the toxic levels for tubificids are considerably above the levels cited by DOUDOROFF & KATZ. Such levels ranged between .01 ppm to 0.7 ppm with varying pH levels and calcium concentrations. ELLIS (1937) reported that some goldfish survived .62 ppm lead as lead nitrate at a pH of 6.8 in hard water but his conclusions are a bit confusing.

Phenate

Sodium pentochlorophenate, (PCP) the water soluble salt of pentachlorophenol is used industrially as a fungicide and as a preservative of wood products. It occurs in industrial effluents in many parts of the country. The toxicity of phenate to tubificids is considerably greater than the toxicity of the two metals investigated. In table 3 the data show an upper level of only 2.0 ppm phenate as compared to the upper levels of 40 ppm and 50 ppm for zinc and lead. The data of table 3 also make obvious the relationship between the pH of the water and the effect of the PCP. It will be noted that as the pH increases the tolerance of tubificids for PCP increases or, perhaps the toxic qualities of the PCP decreases with an increase in the

alkalinity of the solution. In fig. 3 the Tl_m for PCP for pH levels of 7.5, 8.5 and 9.5 double with each increase in the pH, being 0.31 ppm, 0.67 ppm and 1.4 ppm respectively. These data are excellent proof for the point that unless the general quality of the media is determined, toxicity data are inconclusive. It is obvious that failure to control pH would lead to considerable confusion in determination of toxicity levels for PCP. The data show that the estimation of a Tl_m for PCP at pH values of 6.5 and 10.5 was not possible due to all of the worms dying at the lower pH and very few dying at the higher pH. The importance of PCP and pH interaction was determined to be significant at the 0.005 level of significance.

The mechanism of action of PCP has been studied in a number of animals. WEINBACH (1954) showed that PCP caused the uncoupling of oxidative phosphorylation in the white rat and in a snail, *Australorbis glabratus* and may afford a toxic influence to systems in this manner. CRANDALL (1962) reported an increase in the respiration rate of guppies exposed to sublethal concentrations of PCP. These results confirm a syndrome observed in the rabbit by KEHOE, DEICHMANN-GRUEBLER & KITZMILLER (1939) after exposure to PCP. From these studies it would appear that PCP acts on the cellular level to effect respiration and interrupt energy production. This cannot be concluded as the mechanism of action in this study however since no technique to measure this possible mechanism was carried out. However, the worms affected by PCP did not have the mucous precipitate of the lead and zinc treated worms. In addition the much lower levels which produced toxic results with PCP would tend to indicate a more delicate mechanism at work. This could conceivably be a more sensitive biochemical mechanism at the enzyme-substrate level as compared to the less sensitive precipitation action of the metals on the mucous interrupting the physical aspects of gaseous exchange. The influence of pH also points to the involvement of a chemically oriented reaction with PCP due to the great variation of toxic levels produced by variation of the solutions pH. It would appear that the mechanism of action is quite different from that of lead, probably centered at the cellular level in the interruption of a biochemical process instead of the precipitation reaction brought on by the lead forming a physical barrier which blocks gaseous exchange.

Zinc

Zinc is used industrially in many processes dealing with the treatment of metals. It has been known to obliterate populations in streams when it has reached high concentrations, ERICHSEN JONES, (1940). In this study the toxicity of zinc at pH levels of 6.5 and 8.5

was too great to allow the calculation of a Tl_m. This indicates the highly ionizable quality of zinc and the probability of an increase in the effects of the ion in such form. The data in table 4 show that in concentrations below 10 ppm zinc was quite toxic at pH values other than 7.5. At the pH of 7.5 the calculated Tl_m was 46 ppm as shown in fig. 3. The data on the interaction of zinc and pH from table 5 are somewhat puzzling in this respect. The 0.05 level of significance for the ANOV for the interaction of zinc and pH would seem to be less than anticipated. From the data one would expect a greater correlation. However, it is possible that this lowered figure is due to the pH effect on both sides of the neutral area. In the other two toxicants the level of toxicity increased only on one side of the neutral pH. The toxic mechanism of zinc is probably the same as the mechanism for lead. The worms killed by zinc concentrations exhibited the same mucous precipitate as those of the lead experiments. This mucous-zinc complex probably coats the body surface and reduces or completely blocks the exchange of gasses across the body surface. Data are available from studies concerned with the action of zinc on fish which supports this premise in the same manner as the data concerned with the effects of lead, that is a mucous covering of the gills. The effect of zinc as an internal poison can be discounted in these studies because of the time factor as discussed with the lead ion. Zinc is associated with a number of enzymes in some higher animals in which characteristics of zinc toxicity greatly resemble those of its deficiency. This may be due to the interaction of zinc and copper, and the possible reduction in copper uptake due to the mechanisms of uptake being blocked by the presence of zinc or some of its compounds. Such actions by zinc in tubificids however can only be the object of speculation at this time.

According to the review of DOUDOROFF & KATZ (1953) the toxicity levels of zinc as a Tl_m range from 0.3 ppm to 1000 ppm depending upon the form of zinc used, the pH and or hardness of the water and the species of fish involved. However, it seems reasonably safe to state that with zinc present as zinc sulfate in a pH 7.5 buffered modified Knop solution the toxicity of zinc for tubificid worms is much less than the toxicity of zinc for the majority of fish under the same conditions. That is, the worms are much more resistant to zinc levels than are fish in general.

SUMMARY

Tubificid worms have been shown to be quite tolerant to toxic materials such as lead, phenol, and zinc. Their median tolerance

limit (Tl_m) for pH levels in modified Knop solution has been shown to be 5.8 to 9.7. Their Tl_m for lead was 49.0 pp, at pH 6.5 and 27.5 at a pH of 8.5. For sodium pentochlorophenate (PCP) the Tl_m was found to be 0.31 ppm at pH 7.5, 0.67 ppm at pH 8.6 and 1.4 ppm at pH 9.5. The Tl_m determined for zinc was 46.0 ppm at pH 7.5. The interactions of pH and these substances has been examined and discussed, and the importance of definition of the solution has been shown. The mechanism of toxicity for lead and zinc probably is the mucousmetal complex which precipitates on the body wall blocking the exchange of oxygen and carbon dioxide. This is similar to the reaction of these metals observed in the gills of fish. The toxic mechanism of PCP is possibly a biochemical interruption of oxidative phosphorylation. It is not a mucous-effecting mechanism because no mucous precipitate forms as with the metals. In each instance these experiments show tubificids with a quantitative resistance to lead, zinc and phenol greater than similar resistance recorded for various fish species. This provides a basis for quantitative evaluation of the resistant nature of these organisms that may be valuable in future studies of their association to polluted habitats.

Zusammenfassung

Tubificiden haben sich giftigen Materialen wie Blei, Phenyl-säure und Zink gegenüber ziemlich duldsam gezeigt. Ihre Durchschnitts-Duldsamkeitsgrenze (Tl_m) für pH Werte in modifizierter Knop-Lösung sind, wie gezeigt wurde, 5.8 bis 9.7. Ihre Tl_m für Blei war 49.0 ppm bei pH 6.5 und 27.5 bei pH 8.5. Es wurde gefunden, dass das Tl_m für Natrium Pentochlorophenate (PCP) 0.31 ppm bei pH 7.5, 0.67 ppm bei pH 8.5 und 1.4 ppm bei pH 9.5 war. Die festgestellte Tl_m für Zink war 46.0 ppm bei pH 7.5. Die Wechselwirkungen von pH und diesen Substanzen sind geprüft und diskutiert worden und die Wichtigkeit von Erklärung der Auflösung gezeigt. Der Giftigkeits-Mechanismus für Blei und Zink is wahrscheinlich der Schleim-Metall-Komplex, der, den Austausch von Sauerstoff und Kohlenstoffdioxyd blockend, an der Körperwand verflüssigt. Dieses ähnelt der Gegenwirkung der beobachteten Metalle in den Kiemen von Fischen. Der Gift-Mechanismus von PCP ist möglicherweise eine biochemische Unterbrechung sauerstoffartiger Phosphorilation. Es ist kein Schleim-wirkender Mechanismus, weil sich kein Schleim-Präpicitat formt wie bei den Metallen. In jedem Beispiel zeigen diese Experimente bei Tubifiziden einen grösseren quantitativen Widerstand für Blei, Zink und Phenylsäure als ähnliche Widerstände, die bei verschiedenen Fisch-Species registriert wurden. Dieses bildet eine

Basis für quantitative Abschätzung des Widerstandes dieser Organismen, die vielleicht in späteren Studien mit ihrer Verbindung mit verunreinigten Fundorten wertvoll sein kann.

References

CARPENTER, Kathleen - 1927 - The lethal action of soluble metallic salts on fishes. *J. exp. Biol.* 4: 378.

CRANDELL, C. A. - 1960 - The effects of sublethal concentrations of several toxicants to the fish Lebistes reticulatus. Ph.D. thesis. Purdue University.

——— & C. J. GOODNIGHT - 1962 - Effects of sublethal concentrations of several toxicants on growth of the common guppy, Lebistes reticulatus. *Limnol. and Oceanogr.* 7(2): 233–239.

DOUDOROFF, P. - 1951 - Bio-assay methods for the evaluation of acute toxicity of industrial wastes to fish *Sewage and Ind. Wastes* 23(11): 1380.

——— & M. KATZ - 1950 - Critical review of literature on the toxicity of industrial wastes and their components to fish. I. Alkalise, acids and inorganic gasses. *Sewage and Ind. Wastes* 22(11): 1432.

——— & ——— 1953 - Critical review of literature on the toxicity of industrial wastes and their components to fish. II. The metals as salts. *Sewage and Ind. Wastes* 25(7): 802

ELLIS, M. M. - 1937 - Detection and measurement of stream pollution. U. S. Dept. Commerce, Bureau of Fisheries. Bull. 22.

ERICHSEN JONES, J. R. - 1936 - The toxicity of dissolved metallic salts to Polycelis nigra (Muller) and Gammarus pulex (L.). *J. exp. Biol.* XIV(3): 351.

——— - 1940 - A study of the zinc polluted river Ysthwty in North Cardiganshire, Wales. *Ann. appl. Biol.* 27: 368.

——— 1941 - Study of the relative toxicity on anions, with Polycelis nigra as test animal. *J. exp. Biol.* 18(2): 170.

HENDERSON, C. & C. M. TARZWELL - 1957 - Bio-assays for the control of Industrial effluents. *Sewage and Ind. Wastes* 29(9).

KEHOE, R. A., WM. DEICHMANN-GRUEBLER and K. V. KITZMILLER - 1939 - Toxic effects upon rabbits of pentachlorophenol and sodium pentachlorophenate. *J. Indus. Hyg. Toxicol.* 21: 160.

NEEDHAM, J. G., P. S. GALTSOFF, P. S. WELCH & F. E. LUTZ - 1959 - Culture Methods for Invertebrate Animals. S-526 Dover Press pp. 101.

SIMPSON, G. G., A. ROE & R. C. LEWONTIN - 1960 - Quantitative Zoology. Harcourt and Brace, New York, N.Y.

STEPHENSON, J. - 1930 - The Oligochaeta. Clarendon Press, Oxford

WEINBACH, E C. - 1954 - The effect of pentachlorophenol on oxidative phosphorylation. *J. biol. Chem.* 210: 545–550.

WESTFALL, B. A. - 1945 - Coagulation films anoxia in fishes. *Ecology* 26: 283.

WINER, B. J. - 1962 - Statistical Principles in Experimental Design. McGraw-Hill Co., Inc., New York, N.Y.